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Replication of the association of GLT6D1 with aggressive periodontitis in a Sudanese population

Running Title: Genetic variants and AgP in Sudan

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Key words: Aggressive periodontitis, gene polymorphism, Africans

Abstract

Background: Susceptibility to aggressive periodontitis (AgP) is influenced by genetic as well as environmental factors. Studies linking gene variants to AgP have been mainly centred in developed countries with limited data from Africa.

Aim: To investigate whether previously reported candidate gene associations with AgP could be replicated in a population from Sudan.

Methods: The investigation of case-control samples. Cases with AgP (n=132) were identified from patients attending the Periodontal Department in Khartoum Dental Hospital. Age and sex matched controls (n=136), with no evidence of periodontitis, were recruited from the same hospital. Genotyping was performed using the Sequenom MassARRAY iPLEX platform. Analysis focused on gene variants with a minor allele frequency (MAF) > 25% in the Sudanese subjects that had previously been reported to be associated with AgP.

Results: One candidate gene rs1537415 (GLT6D1) was significantly associated with AgP, OR = 1.50 (95% CI 1.04-2.17), $p=0.0295$ (increasing to $p=0.09$ after correction for multiple testing). The association strengthened to OR=1.56 (95% CI 1.15-2.16), $p=0.0042$ when the controls were supplemented with data from the Hap map for the Yoruba in Ibadan (n=147) and remained significant ($p=0.013$) after correction for multiple testing.

Conclusion: The study independently replicated the finding that rs1537415, a variant in glycosyl transferase gene GLT6D1, is associated with AgP and provided the first report of genetic associations with AgP in a Sudanese population.

Clinical Relevance

Scientific rationale for the study: Aggressive periodontitis is more prevalent among Africans than Europeans but studies on potential genetic risk factors for the disease in African populations are lacking.

Principal findings: We independently replicated in a Sudanese population the previous finding in a European study (Schaefer et al. 2010) that a variant in the glycosyl transferase gene GLT6D1 is associated with an increased risk of aggressive periodontitis.

Practical implications: The identification of genetic risk factors may help to facilitate more rational approaches to treatment through a better understanding of disease pathogenesis and an improvement in our ability to identify high-risk groups for periodontitis.

Introduction

Periodontal diseases are opportunistic chronic infections characterized by inflammation and destruction of the supporting tissues of the affected teeth. Periodontal diseases affect up to 90% of the adult population but only about 10% are highly susceptible to severe disease (Kassebaum et al. 2014). Included in those with high susceptibility are individuals who suffer severe destructive periodontal disease in early life and this clinical condition has been defined as aggressive periodontitis (AgP) (Tonetti & Mombelli 1999). AgP is characterized by onset at a young age, rapid periodontal attachment loss with alveolar bone destruction and a tendency to aggregate in families (Tonetti & Mombelli 1999, Llorente & Griffiths 2006). The disease is caused by anaerobic Gram-negative bacteria but disease susceptibility can be influenced by genetic as well as environmental factors (Pihlstrom et al. 2005). Genetically determined variances in the host response to bacterial infections are important determinants for risk of periodontitis (Divaris et al. 2013). Numerous genetic association studies have indicated a possible role for genes encoding proteins associated with the immune response in the susceptibility to periodontitis (Laine et al. 2012). It has been shown that individuals from different populations carry different profiles of genetic variants and there may be substantial geographic differentiation (The 1000 Genomes Project Consortium 2012). Since genotype and allele frequencies can vary between different ethnic populations therefore a genetic risk factor for disease susceptibility in one population may not be a risk factor in another population (Ioannidis 2003, Loos et al. 2005, Meng et al. 2007).

A recent systematic review concluded that the prevalence of AgP varies among different ethnicities and is highest in African populations and their descendants (Susin et al. 2014). The prevalence of AgP in Caucasians residing in north and mid-Europe is 0.1% and in south European populations is higher ~0.5% (Susin et al. 2014). There are higher prevalence rates

for AgP in African populations ranging from 0.5% to 5% (Albandar & Tinoco 2002). A study of high school students in Sudan reported that 3.4% had AgP (Elamin et al. 2010). To date, data linking gene variants to AgP in native African populations is sparse. The greater genetic diversity of African populations present challenges, however, it is important to conduct genetic studies in Africa (Teo et al. 2010). It was hypothesized that variants in candidate genes shown to be associated with AgP in studies in developed countries could also be associated with this condition in African populations. The aim of this study was to investigate whether previously reported candidate gene associations with AgP could be replicated in a population from Sudan.

Subjects and methods

Study participants

Cases and controls were recruited from patients attending the Department of Periodontology Faculty of Dentistry, University of Khartoum and Khartoum Teaching Dental Hospital. Cases with AgP were identified on the basis of a number of criteria including age, severity of interproximal periodontal attachment loss and alveolar bone loss as shown in table 1. Individuals were examined under ideal conditions in a dental chair by an experienced specialist periodontist (N.T.H.). Full-mouth measures of probing pocket depths (PPD) and clinical attachment levels (CAL) were obtained at 6 points per tooth (mesio-buccal, mesio-lingual, disto-buccal, disto-lingual, buccal and lingual). Radiographic examination, which included an orthopantomogram and intraoral views, was also performed for each subject with a clinical diagnosis of AgP to confirm the diagnosis (Table 1). Control subjects were recruited from patients attending non periodontal clinics in the same hospitals who were frequency matched for age, sex and ethnicity, based on ethnic origin by tribe, to those recruited with AgP. The controls in some cases had evidence of gingival inflammation but were free from AgP as well as chronic periodontitis with no PPD or CAL >3mm.

Demographic data including age, gender, tribal affiliation and information regarding smoking were obtained from all potential participants. Subjects were excluded if they were not Sudanese nationals; had diseases or conditions that could pose health risks to the participants or examiners; had medical conditions, identified by self-report, such as diabetes or neutropenias that could influence their periodontal condition; had received a course of antibiotics within the previous month; or had received periodontal treatment before sampling.

The study was approved by the Faculty of Dentistry, University of Khartoum Health Research Ethics Committee (HREC assigned number 1/2008). Informed written consent was obtained from each subject prior to participation in the study.

Blood sample collection and genomic DNA preparation

Four ml of venous blood was collected *via* venipuncture from the antecubital fossa into a 5 ml EDTA tube from each subject diagnosed with AgP and each control. DNA was extracted from blood samples using Qiagen DNeasy, DNA extraction kit (Qiagen, Hilden, Germany) at the Institute for Endemic Diseases, University of Khartoum. Genomic DNA was then quantified by Nanodrop (ND-1000, USA) and stored at -80°C prior to transfer to Northern Ireland for genotyping.

SNP Genotyping

Genotyping was carried out at the genomic core unit at Queen's University Belfast. The genotyping was performed using a commercially available Sequenom MassARRAY iPLEX platform (Sequenom Inc., USA). The assay is based on a primer extension method and consists of an initial locus specific PCR reaction followed by a locus specific primer extension reaction in which an oligonucleotide primer anneals immediately upstream of the polymorphic site being genotyped. The primer and amplified target DNA were incubated with mass-modified dideoxynucleotide terminators. The primer extension was made according to

the sequence of the variant site and is a single complementary mass-modified base. The mass of the extended primer was determined by MALDI-TOF mass spectrometry.

SNPs investigated

The cases and controls were genotyped for SNPs that had been investigated previously for associations with periodontitis. Some SNPs had been investigated in AgP while others had been studied in chronic periodontitis (Tables 2, S1).

In order to investigate possible replication of common variants previously reported to be associated with AgP the following inclusion criteria were applied. The SNPs included in the final statistical analysis had:

- Previously been reported to have an association with AgP.
- Minor allele frequency (MAF) in the Sudanese controls studied of > 25%.
- Genotype call rate of >95%.

Population stratification

The population substructure was estimated using the programme STRUCTURE 2.3.4. The STRUCTURE parameters were 10,000 burn-in periods and 10,000 step chains with up to 3 populations assumed (K1-K3) and 10 replicates were run for each *K*.

Statistical analysis

Based on the number of cases and controls a *post hoc* analysis found the study had 80% power to detect an odds ratio (OR) of 1.7 for an association with AgP. A secondary analysis in which the controls were supplemented with data available from the Hapmap for the Yoruba in Ibadan, Nigeria (International HapMap Project 2014) had 82% power to detect an odds ratio of 1.59 which was the overall OR from the GWAS completed by Schaefer et al. (2010).

Deviation of genotype frequencies from Hardy-Weinberg equilibrium (HWE) was assessed with Haploview 4.2 with a cut off of $p < 0.05$. Only those SNPs that were in HWE were studied further.

Associations with AgP were examined using Haploview 4.2. Pearson's Chi Square was used to test associations between the allele frequencies and AgP with the significance level set at $p < 0.05$. OR and 95% confidence intervals (95% CI) were calculated to report the strength of associations. A Bonferroni correction was applied to take multiple testing into account.

Results

A total of 132 subjects with AgP (mean age $24.8 \pm \text{SD } 5.12$ years) and 136 controls (mean age $23.6 \pm \text{SD } 5.08$ years) were investigated. The majority of the study populations were females (76% cases, 73% controls). All subjects were non-smokers. There were 3 SNPs which met the inclusion criteria for the study (Table 2) and one of these was significantly associated with AgP namely rs1537415 (GLTD6D1).

rs1537415 (GLTD6D1)

The MAF for rs1537415 was 37.8% in AgP cases compared with 28.8% in controls, $p = 0.0295$, OR = 1.50 (95% CI 1.04-2.17). The test for trend was also significant $p = 0.035$. Using a multiplicative model the odds of AgP in an individual who was homozygous for the G allele was 2.25. The association was no longer significant after application of a Bonferroni correction for multiple testing ($p = 0.09$). To further explore the associations data from the Hapmap for the Yoruba in Ibadan (YRI) were used to supplement the control genotypes. The MAF for rs1537415 for the Yoruba ($n = 147$) was 26.9% which was similar to the value for the Sudanese controls (28.8%). This secondary analysis increased the strength of the association with $p = 0.0042$ and a final OR = 1.56 (95% CI 1.15-2.16). The association for

GLT6D1 remained significant after application of a Bonferroni correction for multiple testing ($p=0.013$).

Other variants

The genotype data for those SNPs which did not meet the inclusion criteria are shown in supplementary data (Table S1). Two of these SNPs were significantly associated with AgP in the Sudanese population namely rs16944 (IL1beta -511) and rs4986790 (TLR4 896).

The MAF for rs16944 was 36.8% in AgP cases compared to 46.2% in controls, $p=0.0295$, OR = 0.68 (95% CI 0.48 - 0.96). The test for trend was also significant, $p=0.0323$. The addition of Hapmap genotype data for the YRI ($n=147$), which had a MAF of 39.5% in relation to rs16944, resulted in a weakening of the association for this variant and a loss of significance with $p=0.115$, OR= 0.78 (95% CI 0.58 -1.06).

The rare variant rs4986790 was significantly associated with AgP, $p=0.0053$, OR=2.19 (95% CI 1.21 - 3.83). This was a low frequency variant (MAF AgP cases =16.1%, MAF controls= 8.1%). Analysis with the addition of the Hapmap YRI data ($n=146$), which had a MAF of 4.1%, strengthened the association for rs4986790 with $p<0.0001$, OR= 3.02 (95%CI 1.86 - 4.93).

Population stratification

Analysis of the genotype data, from SNPs which were in HWE, in the programme STRUCTURE divided the study population into three defined clusters which showed within and between cluster homogeneity (Figure S1).

Discussion

The main finding of this study was that a genetic variant in the glycosyl transferase gene GLT6D1 was associated with aggressive periodontitis in a Sudanese population. In the

primary analysis the association became non significant after Bonferroni correction for multiple testing ($p=0.09$). This association was significant after a secondary analysis that incorporated data from the Hapmap for the Yoruba in Ibadan (International HapMap Project 2014) to supplement the controls. Indeed the association of this variant in GLT6D1 was strengthened after incorporating the Hapmap data and remained significant after correction for multiple testing ($p=0.013$). The finding independently replicated the identification of rs1537415 as a candidate genetic variant in one of the few GWAS of AgP (Schaefer et al. 2010) reported to date.

Genome-wide studies are effective at detecting common variants of reasonable effect size, odds ratios greater than 1.2, that contribute to complex traits (Flint & Kendler 2014). The GWAS completed by Schaefer et al. (2010) identified a variant in the glycosyltransferase gene GLT6D1 to be associated with AgP in German cases. The association was replicated in a panel of Dutch generalized and aggressive periodontitis cases. The odds ratio for the association reported by Schaefer et al. (2010) was 1.59 (95% CI 1.36-1.86), which was similar to the result from the final model used in the current study of 1.58. There was enrichment of the minor allele by 9% in the Sudanese population which was broadly similar to that reported by Schaefer et al. (2010) of about 10%. Schaefer et al. (2010) suggested a possible functional role for rs1537415 was the reduction of the binding affinity of GATA-3 which is important for T cell development, homeostasis, activation, proliferation and effector functions of (Zheng & Flavell 1997, Wan 2014).

There are a number of limitations including the small size of the population investigated which indicate the pilot nature of this study. In order to improve the power of the study the controls were supplemented with genotype data from the Hapmap for the Yoruba in Ibadan (International HapMap Project 2014). Despite being a more common condition than in Western Europe the prevalence of AgP in Africa is low and so it was felt that this could be justified. Adjustment for age and gender did not change the outcomes of the primary

analysis, however, it was not possible to adjust for these factors in the larger secondary analysis incorporating Hapmap data. Furthermore, the analyses were not adjusted for possible confounders such as BMI or socioeconomic factors, however, there is limited information that these have been identified as significant risk factors for AgP. Smoking was not a consideration as none of the cases or controls smoked.

To further acknowledge the size of the study we focused principally on common variants with a MAF >25%. This value was chosen to improve the power of replicating any association of the variants being studied with the complex phenotype of AgP (see discussion in Ardlie et al. 2002). The genotyping platform used also provided data on variants which occurred with low frequency in African populations. There were also a number of variants which did not meet the inclusion criteria because previous reports did not find an association with aggressive forms of periodontitis. All the genotype data collected has been provided in a supplementary table so that other researchers can access it. In the analysis limited to SNPS in the supplementary group, rs16944 had a significant association with AgP in the Sudanese population. This variant, however, lost statistical significance when data from the Hapmap was used to supplement the control genotype data. The finding in relation to rs16944 in the sample recruited in Khartoum may therefore represent a false positive. A systematic review of cytokine gene polymorphisms in periodontal disease reported a weak positive association of rs16944 (IL1beta -511) with chronic but not aggressive periodontitis (Nikolopoulos et al. 2008).

A rare variant in the TLR4 gene rs4986790 was also associated with an increased risk of AgP. This finding is in line with previous reports linking rs4986790 with a pro-inflammatory phenotype in African populations (Ferwerda et al. 2007). TLR4 is a critical pathogen recognition receptor for lipopolysaccharide from Gram negative bacteria. Functionally, the rs4986790 variant exhibits an increased TNF- α cytokine response in vitro after stimulation

with LPS and seems to predispose to septic shock in African populations (Ferwerda et al. 2007). In European, but not in African, populations rs4986790 is in linkage with another rare variant rs4986791 and this haplotype has little or no effect on responsiveness to LPS and the susceptibility to infections. It is probable that the findings of the present study represent a false positive association for the TLR4 SNP and this is supported by the contradictory reports of two European studies one reporting an increased (Brett et al. 2005) and the other a decreased risk of AgP (James et al. 2007) associated with this variant. Both the older studies as well as the current study were not adequately powered to identify a true association for this rare variant.

Genetic factors have been suggested to play a role in aggressive periodontitis but studies have been mainly limited to developed countries. The majority of GWAS have been based on European populations. GWA platforms have been designed for optimal use in European populations and are therefore less sensitive in non-European populations (Fu et al. 2011). However, although there are some population specific risk variants it is also the case that genetic variants identified with increased risk of complex traits, such as type 2 diabetes in European populations, have also been shown to be associated with increased risk in diverse racial and ethnic groups (Waters et al. 2010). It is acknowledged that there is great genetic diversity in African populations (DeGiorgio et al. 2009). There are more than 2000 distinct ethnic groups in Africa with evidence that these correlate with genetic differences.

Accordingly the levels of population genetic structure are greater in Africa than in other parts of the world (Teo et al. 2010). In the current study population structure analysis using all the genotype data showed the presence of three distinct but homogenous clusters which mapped to the locally well recognized tribal groupings (Excoffier et al. 1991, Cavalli-Sforza 1997). There was no evidence of significant differences in genotype distribution in these clusters, however, the numbers in each cluster were small. It remains a possibility that we could not fully account for the population structure in the genotype analysis and this may have been a factor in the identification of departure from HWE in 3 variants. While

population structure is a problem in such studies there are advantages to genetic studies in African populations. The low levels of linkage disequilibrium mean that a replication confirmed using African data may be of particular value as it is more likely to indicate a causal variant (Teo et al. 2010). Further studies in African populations with larger sample sizes and correction for population structure may be useful in fine mapping causal variants which have a role the complex pathophysiology of periodontitis.

Conclusion

This is the first study that validates the association of a variant in GLT6D1 with aggressive periodontitis in a population with a different ancestral background to North-Western Europeans. It is also the first report of genetic associations with aggressive periodontitis in a Sudanese population. The study provides pilot data that could be used to design larger studies to explore the genetic links to periodontal disease.

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Age (years)	Number of teeth	Interproximal attachment loss (mm)	Interproximal bone loss (mm)
<20	2 (non adjacent teeth)	>4	>6
<25	3	>5	>7
<30	5	>5	>7
<35	7	>5	>7

Table 1. Criteria for diagnosis of aggressive periodontitis. Adapted from Hodge et al. 2000.

Genetic variant	Historical study type Authors	HWE <i>p</i> value	% genotyped	MAF AgP	MAF Control	Unadjusted <i>p</i>
rs4251961 (IL1 RN)	Association AgP=47; Control= 97 Japan Tai et al. 2002	0.15	98.1	30.8	26.9	0.33
rs10735810 (VitD Fok 1)	Meta-analysis Chen et al. 2012	0.29	97.4	22.5	26.3	0.31
rs1537415 (GLT6D1)	GWAS Schaefer et al. 2010	0.39	97.0	37.8	28.8	0.0295

Table 2. Single nucleotide polymorphisms included in the analysis of aggressive periodontitis in a Sudanese population. These variants had been shown to have an association with AgP in the historical studies outlined. In relation to 'Type of study' specific details of numbers of subjects involved and country of origin have only been given for association studies.

Supplementary data

Genetic variant	Historical study type Authors	HWE <i>p</i> value	% genotype d	MAF AgP	MAF Control	Unadjusted <i>p</i>
rs1800587 (IL1alpha-889)	Meta-analysis Nikolopoulos et al. 2008	0.19	98.1	36.4	36.5	0.99
rs17561 (IL1alpha 4845)	Meta-analysis Mao et al. 2013	0.11	98.5	24.4	21.6	0.44
rs16944 (IL1beta -511)	Meta-analysis Nikolopoulos et al. 2008	0.69	97.8	36.8	46.2	0.0295
rs1800872 (IL10 -592)	Meta-analysis Zong et al. 2012	0.27	97.8	41.5	40.9	0.89
rs763780 (IL17F)	Association AgP=45; CP= 85;Control=72 Brazil Saraiva et al. 2013	0.28	97.8	4.3	6.0	0.38
rs7975232 (VITD Apa1)	Meta-analysis Chen et al. 2012	0.41	98.5	27.7	32.0	0.28
rs1544410 (VIT D Bsm1)	Meta-analysis Chen et al. 2012	0.24	98.9	42.0	41.7	0.95
rs731236 (VIT D 16Taq1)	Meta-analysis Chen et al. 2012	0.38	98.5	45.4	40.2	0.22
rs1800469 (TGFbeta -	Association AgP=172; CP= 147;Control=	0.07	98.5	32.7	33.8	0.78

509)	303 Brazil Kobayashi et al. 2009					
rs1982037 (TGFbeta 869)	Association Kobayashi et al. 2009	0.94	85.8	10.7	5.8	0.059
rs1495741 (NAT2)	Association Severe =74; Moderate = 48; Health= 29 Germany Kocher et al. 2002 *	0.43	91.4	22.0	21.5	0.90
rs1872426 (OPG)	Association AgP=99; Control= 89 Japan Soedarsono et al. 2006 *	0.58	95.1	21.4	22.4	0.79
rs2277438 (RANK L)	Association Soedarsono et al. 2006 *	0.40	94.0	18.5	18.5	0.99
rs3795391 (S100a)	Association AgP=139; Control= 88 China Sun et al. 2011	0.89	98.9	5.0	4.5	0.81
rs4986790 (TLR4 896)	Meta-analysis Ozturk et al. 2009	0.57	95.1	16.1	8.1	0.0053
rs6681231 (COX-2)	Association AgP=532; CP= 1052; Control= 2873 Germany/Holland Schaefer et al. 2010	0.33	81.6	31.6	31.3	0.96

Table S1. Single nucleotide polymorphisms which did not meet the inclusion criteria for statistical analysis of aggressive periodontitis in a Sudanese population. In relation to 'historical study' specific details of numbers of subjects involved and country of origin have only been given for association studies. Two variants rs3795391 (S100a) and rs6681231 (COX-2) were shown to have an association with AgP whereas all other SNPs investigated

either had an association with chronic periodontitis (CP) or no association with AgP or CP in the historical studies cited. There were 3 variants rs2227306 (IL8 -781), rs2275913 (IL17 A) and rs1360590 (CDKN2BAS) which showed departure from HWE and were not investigated further.

* The studies cited did not report on the specific SNP genotyped in the current study but on another variant in the gene.

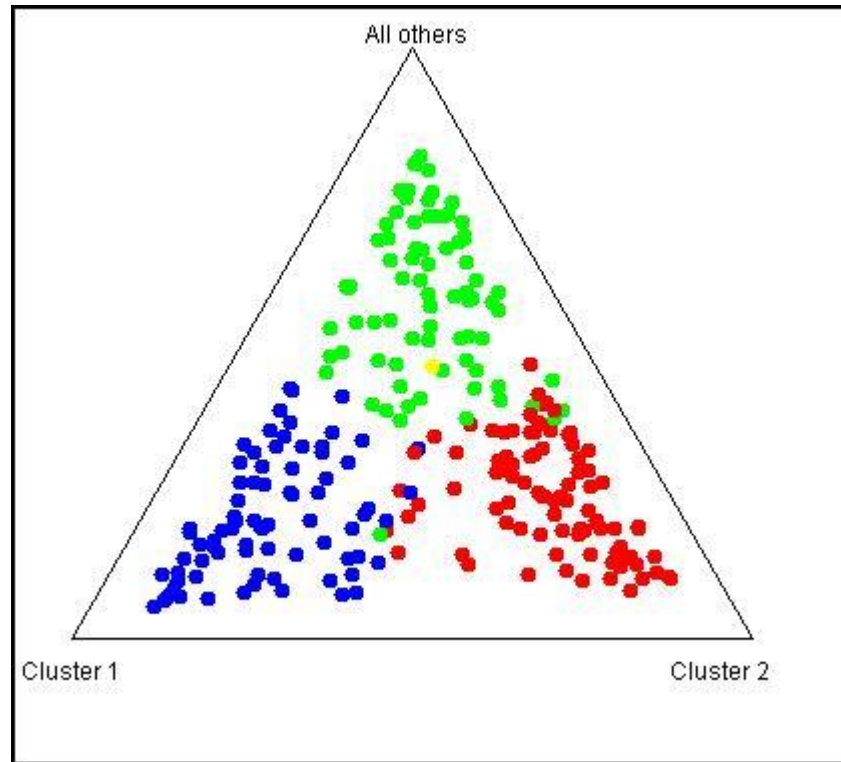


Figure S1. Plotted triangle showing population substructure of aggressive periodontitis cases and controls identified by the programme STRUCTURE. Analysis reveals presence of three different but homogenous substructures.

Supplementary references

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